

N° d'enregistrement
au C.N.R.S. AO 5169

ARTICLE PRINCIPAL
Recouvrant en partie
LA THESE DE DOCTORAT ES SCIENCES NATURELLES

présentée

A LA FACULTE DES SCIENCES
de PARIS
—
par

Monsieur Pierre YOT

pour obtenir
le grade de Docteur Es-Sciences

Sujet de la Thèse : "Estérification Enzymatique du RNA du Virus
de la Mosaïque Jaune du Navet par la Valine"
soutenue le 21 Décembre 1970 devant la Commission d'Examen

MM. GROS Président
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Valine-Specific tRNA-like Structure in Turnip Yellow Mosaic Virus RNA*

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Communicated by Paul Berg, August 17, 1970

Abstract. The 3' terminal nucleotide of turnip yellow mosaic virus (TYMV) RNA (23-25 S) may be esterified with valine in the presence of ATP and an enzyme preparation from *Escherichia coli*. The nucleotide composition near the valine-binding site is different for TYMV RNA and tRNA^{Val} from cabbage, as shown by comparison of the valine adducts of nucleotides labeled with radioactive valine in T₁ RNase digests. Consequently, host tRNA^{Val} is not involved in the observed charging of TYMV RNA with valine. The TYMV RNA appears to have a tRNA-like structure, at or near its 3' end, that is recognized by three different enzymes which specifically catalyze reactions involving tRNA.

We have recently shown that RNA extracted from Turnip Yellow Mosaic Virus (TYMV) binds valine when incubated with ATP and cell-free extracts from *Escherichia coli* that are devoid of nucleic acids. The amino acid is bound by an ester linkage to the 3' terminal nucleotide of the RNA molecule, in a manner analogous to that encountered in aminoacyl-tRNAs.¹ Purified *E. coli* valyl-tRNA synthetase catalyses this reaction, but only after the RNA isolated from TYMV has had an AMP residue linked to its 3' end, from an ATP donor, by tRNA nucleotidyltransferase (EC 2.7.7.25; manuscript in preparation).

In our previous paper,¹ we argued against the possibility that valine is bound to host tRNA^{Val} entrapped within the virus particle. We now present experiments that further confirm our conclusion that valine is indeed bound to TYMV RNA. Our results also exclude the existence of a covalent linkage between host tRNA^{Val}, or part of its structure and the 3' end of TYMV RNA. All of our data suggest that the viral RNA bears a tRNA^{Val}-like structure in the vicinity of its 3' terminus.

Methods and Materials. Extraction of RNA from TYMV: TYMV, isolated from infected cabbage (*Brassica chinensis*) leaves, was kindly supplied to us by Dr. L. Bosch (Leiden), Dr. J. M. Bové (Versailles), and Dr. L. Hirth (Strasbourg). RNA was extracted from the virus particles by phenol treatment² in the presence of bentonite,³ and stored at -30°C in 0.01 M sodium cacodylate, pH 6.

Extraction of tRNA from Chinese cabbage: RNA was extracted from uninfected cabbage leaves by the phenol method, in the presence of 0.01 M Tris (pH 7.4)-0.125 mM EDTA-1% sodium dodecylsulfate, and precipitated by ethanol. The RNA that is soluble in 1.5 M NaCl was isolated, incubated with DNase, and chromato-

graphed on DEAE-cellulose in 0.01 M Tris, pH 7.4; the nucleic acids were eluted with 0.6 M KCl.

DEAE-enzyme preparations from *E. coli*: The 150,000 $\times g$ supernatant of *E. coli* (MRE 600) was freed of nucleic acids by chromatography on DEAE-cellulose equilibrated with 0.01 M Tris, pH 7.4–0.1 mM β -mercaptoethanol. The proteins eluting between 0.1–0.25 M KCl were concentrated by the addition of 90% ammonium sulfate; they were dissolved in 0.01 M Tris, pH 7.4–0.1 mM β -mercaptoethanol, dialyzed against this solution, and stored at -30°C .

Esterification of valine to TYMV RNA and to tRNA: Unless otherwise stated, incubation mixtures contained in 50 μl : 2.5 μmol Tris (pH 7.4), 0.5 μmol MgCl_2 , 0.5 μmol dithiothreitol, 0.1 μmol ATP, 20 μg of DEAE-enzyme preparation, 15–20 μg of (*E. coli*) tRNA, (cabbage) tRNA, or TYMV RNA as indicated, and either 8 nmol of [^3H]valine (500 Ci/mol) or 1 nmol of [^{14}C]valine (109 Ci/mol). After incubation for 20 min at 30°C , the reactions were stopped by the addition of 10% cold trichloroacetic acid; the resulting precipitates were collected on Millipore filters and counted in Bray's solution. For preparative purposes the incubation mixtures were scaled up, and the labeled product was recovered by phenol extraction of the samples after incubation. The extent of aminoacylation of tRNAs (*E. coli* and cabbage) were 1.8 and 0.5 pmol valine/ μg of tRNA, respectively, and that of TYMV RNA was as indicated.

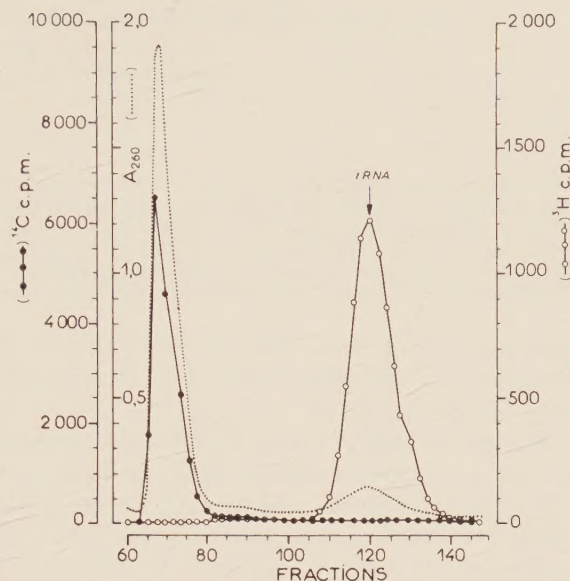
Purified *E. coli* enzymes: Valyl-tRNA synthetase (90% pure), free of all other aminoacyl-tRNA synthetases, was a generous gift of Dr. M. Yaniv and Mrs. A. Chestier.⁴ The 50%-pure tRNA nucleotidyltransferase was kindly donated to us by Miss D. Carré and Mr. S. Litvak.⁵ Purified *N*-acylaminoacyl-tRNA hydrolase,⁶ prepared by P. Yot and Mrs. D. Paulin, was also used.

Results. Sephadex G-75 behavior of TYMV RNA preparations charged with [^{14}C]valine: It has already been reported that when TYMV RNA was incubated in the presence of *E. coli* DEAE-enzyme preparations, ATP, and [^{14}C]valine, and the product analyzed by sucrose gradient centrifugation, a significant amount of the radioactive material sedimented at 23–25S.¹ This high molecular weight material was examined further by filtration through Sephadex G-75, and compared to labeled (*E. coli* or cabbage) [^3H]valyl-tRNA added as marker. Fig. 1 demonstrates that, as expected, the ^{14}C -labeled RNA was not retarded by the column in comparison to [^3H]valyl-tRNA.

Acceptor activity of TYMV RNA in the presence of valyl-tRNA synthetase: Previous results indicated that when the *E. coli* DEAE-enzymes were incubated with ATP and fifteen ^{14}C -labeled amino acids, only valine was esterified to TYMV RNA preparations.¹ With identical conditions, virtually all amino acids were linked to (*E. coli*) tRNA, and more than half of them to (cabbage) tRNA. Therefore, the DEAE-enzyme preparation contained several aminoacyl-tRNA synthetases. To determine whether valyl-tRNA synthetase was responsible for the attachment of valine to TYMV RNA, the enzyme preparations were chromatographed on DEAE-cellulose using a KCl gradient. All the fractions eluting between 0.1–0.3 M KCl were assayed for their ability to transfer valine to tRNA and TYMV RNA. We found that those fractions that promoted the transfer of valine to tRNA also linked valine to TYMV RNA preparations. This suggests that, as is the case with tRNA, the valyl-tRNA synthetase attached valine, by an ester linkage, to the terminal adenosine of TYMV RNA.¹

To further verify this point, highly purified valyl-tRNA synthetase was used in place of the DEAE-enzyme preparation; surprisingly, under these conditions, valine was not charged to the TYMV RNA. However, if tRNA nucleotidyl-

FIG. 1. Sephadex G-75 filtration of TYMV [^{14}C]valyl-RNA and of (*E. coli*) [^3H]valyl-tRNA. A sample, containing in 1 ml: 10 μmol sodium cacodylate, pH 6, 0.1 mmol NaCl, 1.5 mg TYMV [^{14}C]valyl-RNA (charged with 240 pmol of [^{14}C]valine) and 300 μg (*E. coli*) [^3H]valyl-tRNA, was applied to a Sephadex G-75 column (2×160 cm) equilibrated with 0.01 M sodium-cacodylate, pH 6, and 0.1 M NaCl. The A_{260} was determined directly on the fractions collected, and aliquots were removed for radioactive measurements.



transferase was included in the reaction mixtures, AMP was incorporated at the 3' end of the RNA, and valine was then attached to TYMV RNA. The details of these experiments will be reported elsewhere by Litvak and Carré (in preparation). Table 1 shows that, although valyl-tRNA synthetase sufficed to charge valine to either (*E. coli* or cabbage)tRNA, tRNA nucleotidyltransferase was necessary to promote this binding in the case of RNA extracted from TYMV.

TABLE 1. Acceptor activity of various RNAs for [^{14}C]valine.

Enzymes added	<i>E. coli</i> tRNA (cpm/ μg RNA)	Cabbage tRNA (cpm/ μg RNA)	TYMV RNA (cpm/ μg RNA)
DEAE-enzyme preparation	425	340	56
Valyl-tRNA synthetase	406	378	5
tRNA nucleotidyltransferase, and valyl- tRNA synthetase	375	279	53

Where indicated, 0.5 μg of valyl-tRNA synthetase and 1 μg of tRNA nucleotidyltransferase were added in place of the DEAE-enzyme preparation; (cabbage) tRNA was further purified on Sephadex G-75.

In other experiments not reported here, we have found that partly purified valyl-tRNA synthetases from yeast and rat liver were also active in esterifying valine to RNA extracted from TYMV. This, and the established fact that valine charging can occur with heterologous systems, suggests that (*E. coli*) valyl-tRNA synthetase is charging valine specifically to a tRNA^{val}-like structure.

Effect of hydrolase: At least two enzymes specific for tRNA recognize RNA extracted from TYMV: valyl-tRNA synthetase and tRNA nucleotidyltransferase. We examined the effect of a third enzyme specific for tRNA, the hydrolase, which cleaves the ester linkage between *N*-acylamino acids and tRNA.⁷ Accordingly, TYMV [^{14}C]valyl-RNA was acetylated and incubated in the

TABLE 2. *Effect of N-acylaminoacyl-tRNA hydrolase on TYMV acetylvalyl-RNA.*

Enzyme	(% hydrolysis)	
	acetylvalyl-RNA TYMV	acetylvalyl-tRNA <i>E. coli</i>
—	3.7	3.8
+	67	88

Acetyl-[^{14}C]valyl-tRNA (*E. coli*) and TYMV acetyl-[^{14}C]valyl-RNA were prepared⁸; in the case of the latter substrate, the reaction was performed in the presence of 0.25 M potassium acetate, pH 5. Incubations contained, in 20 μl : 10 μmol sodium barbital-acetic acid, pH 8.6, 4 μg (8 units) of purified hydrolase, and either 50 μg of TYMV acetyl-[^{14}C]valyl-RNA (charged with 8 pmol of [^{14}C]valine) or 35 μg of acetyl-[^{14}C]valyl-tRNA. After 30 min at 37°C, the acetyl-[^{14}C]valine formed was separated from the substrate by paper electrophoresis.⁷

presence of hydrolase. The results (Table 2) show that acetylvaline was liberated under conditions identical to those required to liberate this compound from acetylvalyl-tRNA.

Absence of tRNA^{Val} from TYMV RNA preparations: We have already commented upon the possibility that the acceptor activity of TYMV RNA for valine might be due to contaminating host tRNA^{Val} entrapped within the virus particle.¹ Indeed, avian myeloblastosis virus is known to contain several tRNA molecules within it.^{9, 10} However, in the case of TYMV RNA, the following observations argue against such a hypothesis¹: (1) the amount of valine esterified per mol of RNA (molecular weight: 2×10^6) never exceeds one to one; (2) only valine, of fifteen amino acids tested, is attached to TYMV RNA; (3) (cabbage) valyl-tRNA shows no propensity to form complexes with TYMV RNA; (4) a small amount of slowly-sedimenting material that bears valine has a higher sedimentation constant in sucrose gradients than that of valyl-tRNA and probably originates from some degradation of TYMV RNA during incubation.

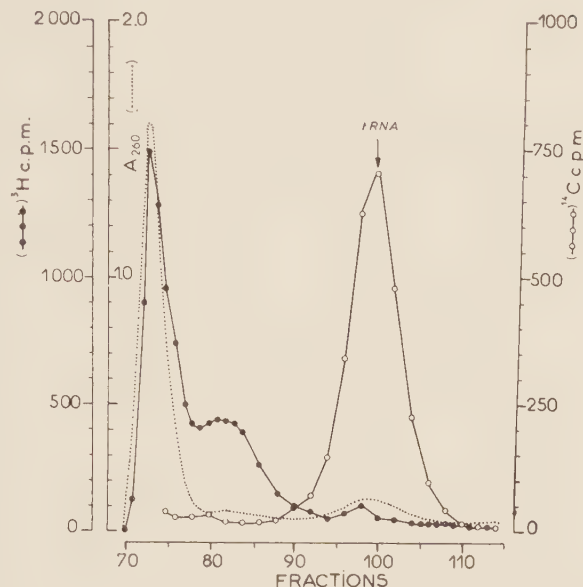
These arguments do not entirely exclude the possibility that host tRNA^{Val} entrapped within the virus particle might somehow be strongly bound to the viral RNA. Various types of linkages could be responsible for such an interaction, for example, protein-mediated linkages, ionic bonds, and hydrogen bonds.

However, all attempts to dissociate such a putative complex have not revealed the existence of a chargeable tRNA species in the TYMV preparations. For example, sedimentation of charged TYMV RNA in sucrose gradients containing 0.5% sodium dodecylsulfate, or 0.01 M EDTA, did not separate the labeled valine from the high molecular weight TYMV RNA. Moreover, filtration of TYMV [^{14}C]valyl-RNA through Sephadex G-100 in 8 M urea does not yield a component with properties of valyl-tRNA. Fig. 2 shows an even more stringent experiment¹¹: filtration of a mixture of [^{14}C]valyl-tRNA (cabbage) and TYMV [^3H]valyl-RNA over Sephadex G-75, after the mixture was treated with dimethylsulfoxide (DMSO) followed by formaldehyde, did not give any ^3H label accompanying the [^{14}C]valyl-tRNA.

As a result of these experiments, it appears most unlikely that the viral RNA is contaminated by host tRNA^{Val}.

T₁ RNase digests of TYMV RNA charged with [^3H]valine: Conceivably, host tRNA^{Val} might have been linked covalently to TYMV RNA by a ligase. If this were so, valine would be linked to a sequence identical to that of host tRNA^{Val}. To examine this possibility, TYMV RNA esterified with [^3H]valine and

FIG. 2. Sephadex G-75 filtration of TYMV [^3H]valyl-RNA and of (cabbage) [^{14}C]valyl-tRNA after DMSO and formaldehyde treatment. The incubation mixture contained in 1.4 ml: 4.5 μmol sodium cacodylate, pH 6, 1 ml DMSO, 960 μg of TYMV [^3H]valyl-tRNA (charged with 380 pmol of [^3H]valine) and 100 μg of (cabbage) [^{14}C]valyl-tRNA. After 10 min at room temperature, the nucleic acids were precipitated with cold ethanol. The pellet recovered after centrifugation was dissolved in 1 ml of water containing formaldehyde (1%), sodium cacodylate (0.01 M at pH 6) and NaCl (0.1 M) and kept 10 min at 0°C . It was applied onto a Sephadex G-75 column (1.9×150 cm) and analyzed as in Fig. 1.



(cabbage) [^{14}C]valyl-tRNA were incubated together with T_1 RNase (guanylo-ribonuclease). The resulting material was applied to a DEAE-cellulose column in the presence of urea,¹² and the products were eluted by an ammonium acetate gradient. Fig. 3 shows that two major [^{14}C]valyl-oligonucleotide peaks (*d* and *e*) were recovered from the cabbage valyl-tRNA, indicating that there are at least two different isoacceptor tRNAs for valine in cabbage. On the other hand, only one [^3H]valyl-oligonucleotide peak (*c*) was observed with TYMV valyl-RNA, and it was distinct from the two [^{14}C]valyl-oligonucleotides originating from valyl-tRNA.

Fig. 3 also shows that the T_1 RNase digest of (*E. coli*) [^{14}C]valyl-tRNA gives two peaks (*a* and *b*). These presumably correspond to two [^{14}C]valyl-oligonucleotides of known sequence (5 and 13 nucleotides, respectively) resulting from at least two valine acceptor tRNAs reported for *E. coli* (ref. 13, and M. Yaniv, personal communication).

Consequently, we conclude that host tRNA^{Val} neither contaminates, nor is linked to, TYMV RNA, and that the valine acceptor activity of TYMV RNA constitutes an intrinsic property of the viral RNA itself.

Degradation of TYMV RNA: If TYMV RNA is maintained for 10 min at 80°C and rapidly cooled, the resulting material appears more heterogeneous and sediments between 23 and 10S on sucrose gradients. This heated TYMV RNA still retains its full capacity to accept valine. The integrity of 23S TYMV RNA is therefore not a prerequisite for charging activity. The behavior of TYMV [^3H]valyl-RNA, obtained by charging valine on heated TYMV RNA, was analyzed both by sucrose gradient centrifugation and on Sephadex G-75. In the sucrose gradient centrifugation (not shown here), the radioactive product sedimented slowly and most of the RNA was close to the top of the gradient. When the same material was analyzed by Sephadex G-75 filtration (Fig. 4),

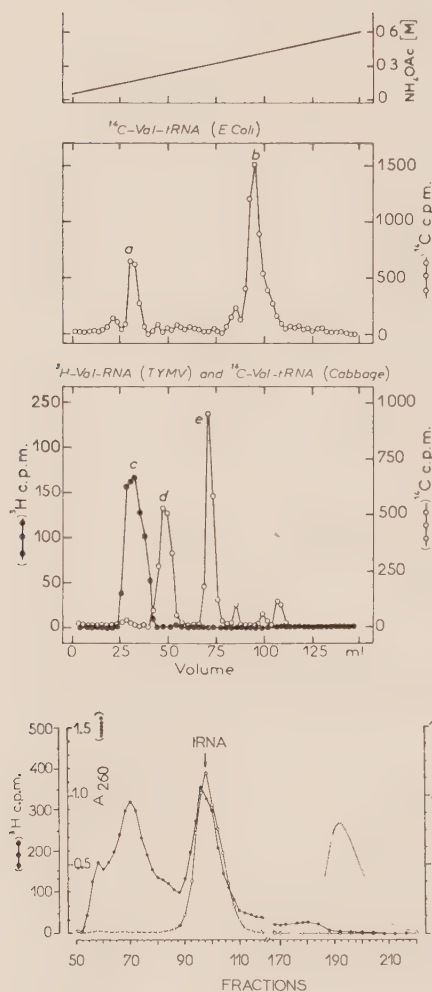
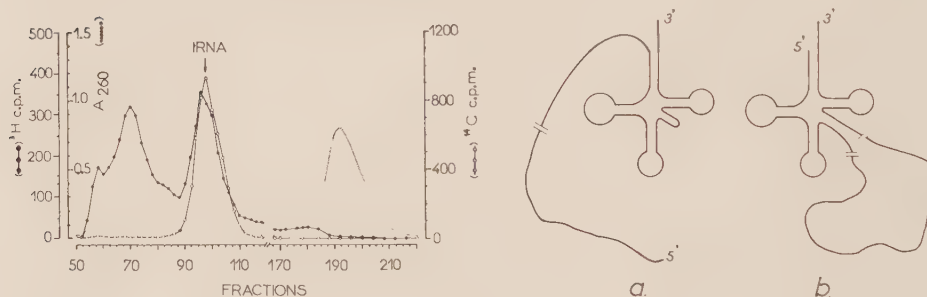


FIG. 3. DEAE-cellulose chromatography of T_1 RNase digests of various RNAs charged with valine. The incubation was carried out in the presence of 0.2 M Tris, pH 7.4, 0.02 M EDTA and 0.25 units/ μg RNA of T_1 RNase (Sankyo Co. Ltd). The total volume was 0.5 ml, and included 360 μg of (*E. coli*) [^{14}C]valyl-tRNA, or the volume was 1.5 ml and contained 350 μg of [^{14}C]valyl-tRNA and 2 mg of [^3H]valyl-RNA (charged with 760 pmol of [^3H]valine) in the case of cabbage and TYMV. After 15 min at 37°C , the samples were adjusted to 0.05 M ammonium acetate, pH 5.5, and 7 M urea, and layered onto a DEAE-cellulose column (0.9×15 cm) equilibrated with 0.05 M ammonium acetate, pH 5.5 and 7 M urea. After the column was washed, a gradient of 0.05 M–0.6 M ammonium acetate, pH 5.5 in 7 M urea was applied (total volume = 150 ml). 1-ml aliquots were removed for counting in 1 ml formic acid and 10 ml Bray's solution. The results shown here were consistent with those obtained when TYMV [^3H]valyl-RNA and (cabbage) [^{14}C]valyl-tRNA were examined separately after T_1 RNase digestion.



(Left) FIG. 4. Sephadex G-75 filtration of TYMV [^3H]valyl-RNA prepared with preheated TYMV RNA and (cabbage) [^{14}C]valyl-tRNA. TYMV RNA (2 mg/ml in 0.01 M sodium cacodylate, pH 6) was heated 10 min at 80°C and rapidly cooled. It was then used as an acceptor of [^3H]valine with a DEAE-enzyme preparation. After phenol extraction and ethanol precipitation, 1.8 mg of this TYMV [^3H]valyl-RNA (charged with 230 pmol of [^3H]valine) was dissolved in 1.3 ml of a solution containing: 13 μmol sodium cacodylate, pH 6, 0.13 mmol NaCl, and 508 μg of (cabbage) [^{14}C]valyl-tRNA. The mixture was applied to a Sephadex G-75 column as described in Fig. 1.

(Right) FIG. 5. Possible models for TYMV RNA^{Val}.

most of the 260 nm-absorbing material appeared as ethanol-precipitable oligonucleotides greatly retarded on the column; the radioactive material was resolved into two predominant populations of molecules, one of which was eluted shortly after the void volume, and the other nearly overlapped [^{14}C]valyl-tRNA, included as marker.

To verify that TYMV [^3H]valyl-RNA, obtained with heated TYMV RNA, still only gave rise to one [^3H]valyl-oligonucleotide after T_1 RNase digestion, we performed an experiment as already described in Fig. 3; the behavior of the

only radioactive fragment obtained in this case was identical to that observed previously (Fig. 3, peak *c*).

The two populations of [^3H]valyl-RNA (Fig. 4) result from partial degradation of high molecular weight TYMV [^3H]valyl-RNA, presumably due to nucleases contained in our DEAE-enzyme preparations. Since the two populations obtained from TYMV valyl-RNA were protected from further cleavage, it is tempting to postulate that their secondary structure is responsible for this protection. This hypothesis is reinforced by the fact that under similar conditions, heated or nonheated (*E. coli* or cabbage) tRNA^{Val} appears not to be degraded by the DEAE-enzyme preparations.

Discussion. The results reported in this paper raise the question of whether the RNA extracted from TYMV bears a tRNA-like structure. This structure, which is recognized by three enzymes specific for tRNA, appears to be an integral part of the viral RNA.

Conceivably the tRNA-like structure is located in the vicinity of the 3' terminus of the viral RNA (Fig. 5*a*) and is composed solely of sequences in this extremity. Alternatively, the primary structure that imparts the valine-acceptor activity is distributed between the 5' and the 3' ends of the RNA molecule (Fig. 5*b*), and only by hydrogen bonding between these regions is a tRNA-like structure formed; this model is compatible with a circular structure of the viral RNA.¹⁴

Although we cannot now choose between these two structures, the following experiment is more consistent with the first formulation. The RNA fragment bearing [^3H]valine that sedimented close to 4 S (Fig. 4) was mixed with (cabbage) [^{14}C]valyl-tRNA and, after treatment with DMSO and formaldehyde, the mixture was applied to a Sephadex G-75 column. The [^3H]valyl-RNA fragment, thus treated, appeared with the [^{14}C]valyl-tRNA marker. If the structure shown in Fig. 5*b* was correct, treatment of this [^3H]valyl-RNA fragment with DMSO and formaldehyde should have led to the formation of two shorter fragments; the one bearing [^3H]valine would have been eluted from the column considerably later than the [^{14}C]valyl-tRNA that had been identically treated.

Our results, and the fact that all bacteriophage and viral RNAs examined to date contain identical sequences (-CpCpA or -CpC) at their 3' termini, suggest that under appropriate conditions these RNAs could also accept an amino acid. It is of course conceivable that even if these RNAs had lost their charging capacity as a result of mutations, they could nevertheless have retained the vestige of a tRNA-like structure. In this case, only a knowledge of the sequence of about the last 50 nucleotides at the 3' terminus of these RNAs would reveal the existence of such a vestige.

We thank Dr. P. Berg for extensive corrections and clarification of the manuscript, Drs. A. M. Michelson and G. Bernardi for critical reading of the manuscript, Dr. F. Galibert for his help with the Sephadex G-75 filtration, and Miss M. F. Poulet and Mr. J. P. Dumas for technical assistance.

Abbreviations: TYMV, turnip yellow mosaic virus; DMSO, dimethylsulfoxide.

*Supported by grants from the Délégation Générale à la Recherche Scientifique et Technique, the Commissariat à l'Energie Atomique (Saclay), and the Centre National de la Recherche Scientifique, Département de Biologie du Centre Nucléaire de Cronenbourg.

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